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A chemically synthesized pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers

David Roise[1,3], Suzanna J.Horvath[2], John M.Tomich[2], John H.Richards[2] and Gottfried Schatz[1]

[1]Biocenter, University of Basel, CH-4056 Basel, Switzerland, and [2]The Braun Laboratories of Molecular Biology, California Institute of Technology, Pasadena, CA 91125, USA

[3]Fellow of The Jane Coffin Childs Memorial Fund for Medical Research

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Subunit IV of yeast cytochrome oxidase is made in the cytoplasm with a transient pre-sequence of 25 amino acids which is removed upon import of the protein into mitochondria. To study the function of this cleavable pre-sequence in mitochondrial protein import, three peptides representing 15, 25 or 33 amino-terminal residues of the subunit IV precursor were chemically synthesized. All three peptides were freely soluble in aqueous buffers, yet inserted spontaneously from an aqueous subphase into phospholipid monolayers up to an extrapolated limiting monolayer pressure of 40–50 mN/m. The two longer peptides also caused disruption of unilamellar liposomes. This effect was increased by a diffusion potential, negative inside the liposomes, and decreased by a diffusion potential of opposite polarity. The peptides, particularly the two longer ones, also uncoupled respiratory control of isolated yeast mitochondria. The 25-residue peptide had little secondary structure in aqueous buffer but became partly α-helical in the presence of detergent micelles. Based on the amino acid sequence of the peptides, a helical structure would have a highly asymmetric distribution of charged and apolar residues and would be surface active. Amphiphilic helicity appears to be a general feature of mitochondrial pre-sequences. We suggest that this feature plays a crucial role in transporting proteins into mitochondria.

Key words: amphiphilic helix/membrane perturbation/mitochondria/pre-sequence/protein import

Introduction

Intracellular sorting of proteins is one of the most fascinating current problems in cellular biology. Proteins made on cytoplasmid polysomes must be distributed specifically to various organelles within the cell, or must be secreted to locations outside the plasma membrane. A central aspect of this process is the transfer of proteins across biological membranes. Since virtually all proteins contain at least some hydrophilic residues, specific mechanisms must exist to transport those residues through the hydrophobic core of phospholipid bilayers.

One approach to the problem of protein sorting is the identification and isolation of components mediating the transfer of proteins across membranes. This approach has already been successful with the endoplasmic reticulum (Meyer *et al.*, 1982; Walter *et al.*, 1984); genetic evidence suggests that this approach should also be applicable to the secretion of proteins from bacteria (Benson *et al.*, 1985) and to the import of proteins into mitochondria (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985).

A second approach is to analyze the transported proteins for structural features which allow them to be translocated across specific biological membranes. The most obvious feature shared by most of these proteins is a transient N-terminal extension ('signal-', 'leader-', or 'pre-' sequence) which is removed proteolytically on the *trans* side of the target membrane (for review, see Wickner and Lodish, 1985). These pre-sequences are indispensable for translocation to occur, but their mechanism of action is not yet clear. Pre-sequences of several eukaryotic secretory and imported mitochondrial proteins (Lingappa *et al.*, 1984; Hurt *et al.*, 1984; Horwich *et al.*, 1985; van Loon *et al.*, 1986) and the pre-sequence of one imported chloroplast protein (van den Broeck *et al.*, 1985) are sufficient to transport attached foreign 'passenger' proteins to and across the appropriate target membranes. Gene fusion and deletion studies indicate, moreover, that some mitochondrial pre-sequences also contain enough information for correctly sorting proteins within mitochondria (Hase *et al.*, 1984; van Loon *et al.*, 1986). It should thus be possible to study some aspects of protein transport using chemically synthesized pre-sequence peptides.

One example of this approach has been the analysis of four chemically synthesized bacterial signal peptides (Briggs and Gierasch, 1984; Briggs *et al.*, 1985). These workers have shown a strong correlation between the helicity of the isolated peptides in an apolar environment, their interaction with lipid model systems and the function of the corresponding pre-sequence *in vivo*. Chemically synthesized pre-peptides of imported mitochondrial proteins have also been reported to inhibit the translocation of authentic mitochondrial precursor proteins into isolated mitochondria (Ito *et al.*, 1985; Gillespie *et al.*, 1985), but the mechanism of this inhibition remains unknown.

In this paper we show that a peptide representing the pre-sequence of cytochrome oxidase subunit IV (an imported mitochondrial protein) can form an amphiphilic helix which is sufficiently surface-active to strongly perturb phospholipid bilayers. This effect is enhanced by a membrane potential of the same polarity as that required for import of the authentic subunit IV precursor into mitochondria. We suggest that this potential-enhanced perturbation of a phospholipid bilayer may be part of the mechanism by which this pre-sequence translocates attached proteins into mitochondria.

Results

Pre-sequence peptides insert into phospholipid monolayers

Subunit IV of yeast cytochrome oxidase is made in the cytoplasm as a precursor containing a 25-residue N-terminal pre-sequence and a 130-residue 'mature' region. The pre-sequence is removed upon import into the mitochondrial inner membrane by a matrix-localized protease (Böhni *et al.*, 1983; Maarse *et al.*, 1984). If the subunit IV pre-sequence is fused to various non-mitochondrial proteins by recombinant DNA methods, the resulting fusion proteins are imported into mitochondria (Hurt *et al.*, 1984; C.Bibus, unpublished).

To study the mechanism of action of this pre-sequence, the following three peptides were synthesized (Figure 1): (i) a 25-residue peptide (p25) which exactly represents the pre-sequence; (ii) a 33-residue peptide (p33) representing the pre-sequence (but lacking the amino-terminal methionine) plus the first nine residues of the mature subunit IV; (iii) a peptide (p15) representing the N-terminal 15 residues of the pre-sequence. All three peptides were highly soluble in aqueous buffers (>20 mg/ml), yet were also extremely surface active: sub-micromolar concentrations caused surface pressures of 10 – 12 mN/m at an air – buffer interface (Figure 2A, points at 0 mN/m initial pressure). In the presence of a monolayer of mitochondrial phospholipids, much larger surface pressures were observed (Figure 2A, points between 15 and 30 mN/m initial pressure). The extrapolated 'limiting pressures' (at which addition of peptides to the subphase would no longer cause any pressure increase) were 43 mN/m for p15, 49 mN/m for p25 and 47 mN/m for p33.

The dependence of the monolayer pressure increase on the concentration of the peptides in the subphase is shown in Figure 2B. At moderate initial pressure (20 mN/m), insertion of the peptides was observed between 10 and 30 nM. Maximum pressure increases were achieved for the two longer peptides at 0.3 μM, and at somewhat higher concentrations for p15. The surface activity of all the peptides was abolished by pre-treatment of peptide solutions with trypsin (1:100, weight trypsin:weight peptide) showing the effect is not due to non-proteinaceous contaminants (not shown).

$\overset{+}{N}H_3$-leu-ser-leu-$\overset{+}{a}$rg-gln-ser-ile-$\overset{+}{a}$rg-phe-phe-$\overset{+}{l}$ys-pro-ala-thr-$\overset{+}{a}$rg-thr- p33
leu-cys-ser-ser-$\overset{+}{a}$rg-tyr-leu-leu-gln-gln-$\overset{+}{l}$ys-pro-val-val-$\overset{+}{l}$ys-thr-ala-$CONH_2$

$\overset{+}{N}H_3$-met-leu-ser-leu-$\overset{+}{a}$rg-gln-ser-ile-$\overset{+}{a}$rg-phe-phe-$\overset{+}{l}$ys-pro-ala-thr-$\overset{+}{a}$rg-thr- p25
leu-cys-ser-ser-$\overset{+}{a}$rg-tyr-leu-leu-$CONH_2$

$\overset{+}{N}H_3$-met-leu-ser-leu-$\overset{+}{a}$rg-gln-ser-ile-$\overset{+}{a}$rg-phe-phe-$\overset{+}{l}$ys-pro-ala-thr-COO^- p15

Fig. 1. Amino acid sequences of the three peptides used in this study. Hydrophobic residues are underlined, and charges are as indicated. In peptide p33, the border between the regions corresponding to the transient pre-sequence and the mature sequence is marked by an arrow.

Pre-sequence peptides disrupt unilamellar phospholipid vesicles in a potential-sensitive manner

The ability of peptides to permeabilize phospholipid bilayers was measured using sonicated unilamellar vesicles containing the self-quenching fluorescent dye, carboxyfluorescein (Weinstein *et al.*, 1977). If this dye is trapped at high concentrations inside vesicles during sonication its fluorescence is quenched. Disruption of the vesicle bilayer thus reveals itself as dequenching of the dye's fluorescence as the dye is diluted into the surrounding medium. This assay was further modified to determine the effects of a membrane potential on the disruption process. When vesicles containing entrapped carboxyfluorescein with K^+ as the counter-cation were treated with valinomycin (a K^+-selective ionophore) and diluted 100-fold into K^+-free buffer, a K^+ diffusion potential was produced (see below for demonstration of potential). Under our conditions, the potential was negative inside the vesicles and, based on the Nernst equation, was ~120 mV. Such a potential is similar in size to the membrane potential measured in rat liver mitochondria under various conditions (Mitchell and Moyle, 1969) and ~3- to 6-fold higher than the potential able to effect import of the ADP/ATP carrier protein into isolated *Neurospora* mitochondria (Pfanner and Neupert, 1985). In an analogous manner, vesicles containing entrapped Na^+ in place of K^+ were treated with valinomycin and diluted into K^+-containing buffer. This created a diffusion potential of the opposite polarity (i.e. positive inside the vesicles) and with a theoretical magnitude in excess of 120 mV. The two types of vesicles were also diluted into buffer whose K^+ concentration was identical to that inside the vesicles; in that case no valinomycin-induced potential would be expected.

Vesicles prepared as above, and treated with various concentrations of p25 and p33, rapidly released their entrapped carboxyfluorescein. A time course of the release induced by p25 is shown in Figure 3. The most rapid release occurred with vesicles having a diffusion potential negative inside (panel A). Vesicles lacking any diffusion potential also rapidly released their entrapped dye, but only at higher peptide concentrations (panel B, C). The slowest release occurred with vesicles having a diffusion potential positive inside (panel D). In the absence of peptide (traces marked 0), all the vesicles were extremely stable over the time course of the experiment.

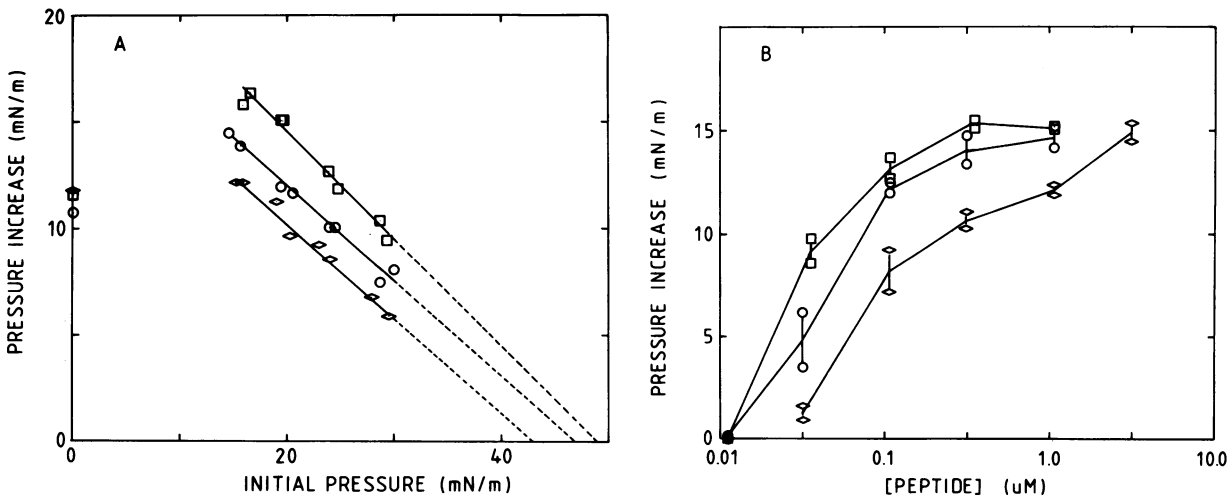

Fig. 2. Interaction of the peptides with a lipid monolayer. Peptide solutions were injected through a monolayer of yeast mitochondrial phospholipids. **(A)** Effect of initial surface pressure on pressure increase at constant peptide concentrations. Points at zero initial surface pressure represent the surface pressure increase in the absence of a lipid monolayer. [p33] = 0.27 μM, [p25] = 0.14 μM, [p15] = 0.44 μM. **(B)** Effect of peptide concentration on surface pressure increase in monolayers. The initial surface pressure of the peptide-free monolayer was 20 mN/m. (○) p33; (□) p25; (◇) p15.

These experiments did not reveal whether release of the entrapped carboxyfluorescein was due to the formation of discrete peptide-induced pores, or whether the peptides caused a general lysis of the liposomes by creating other structural changes in the lipid bilayer. Since electron microscopy revealed gross morphological alterations of the liposomes (cf. below), we conclude that peptide-induced release of entrapped carboxyfluorescein reflects at least partial lysis of the liposomes (see also Discussion). It is not known whether changes in liposome structure (i.e. size increase) could cause changes in fluorescence of the dye remaining trapped within the liposomes. However, since the fluorescence of released carboxyfluorescein is directly proportional to the concentration of the dye under the conditions used here (not shown), we assume a direct relationship between release of dye, fluorescence and lysis of the liposomes.

The concentration dependence of the rate of lysis induced by p25 and p33 is plotted in Figure 4. Rates were arbitrarily measured between 30 and 60 s after addition of vesicles to the peptide solutions. With both peptides the fastest lysis occurred with vesicles having a diffusion potential negative inside. The lytic rates of the two peptides varied ~3- to 5-fold with the polarity of the diffusion potential. Lysis of vesicles by p15 was observed only at much higher peptide concentrations, and was not further studied. Trypsin treatment of the peptides completely abolished their lytic effects on vesicles (not shown).

The membrane potentials induced in K^+-containing vesicles were directly demonstrated with the potential-sensitive cyanine dye, diS-C_3-(5) (Sims *et al.*, 1974). Figure 5 shows the change

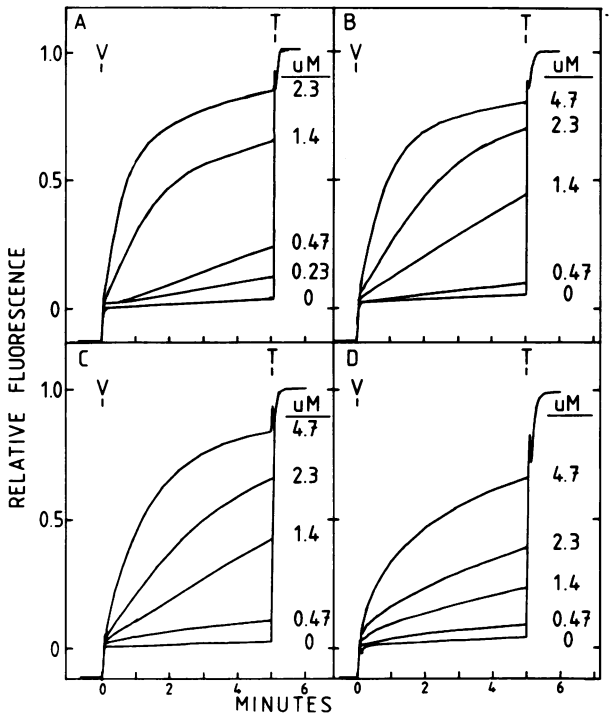

Fig. 3. The peptide p25 induces potential-sensitive release of carboxyfluorescein from phospholipid vesicles. POPC:CL vesicles loaded with carboxyfluorescein and treated with valinomycin were added at the point labeled 'V' to solutions containing the indicated concentrations of p25 in a stirred fluorescence cuvette. Release of carboxyfluorescein fluorescence was followed versus time. Maximum fluorescence (taken as 1.0) was determined after addition of Triton X-100 to 0.1% at the point labeled 'T'. **(A)** K^+-containing vesicles in Na^+ buffer (potential negative inside); **(B)** K^+-containing vesicles in K^+ buffer (no diffusion potential); **(C)** Na^+-containing vesicles in Na^+ buffer (no diffusion potential); **(D)** Na^+-containing vesicles in K^+ buffer (potential positive inside).

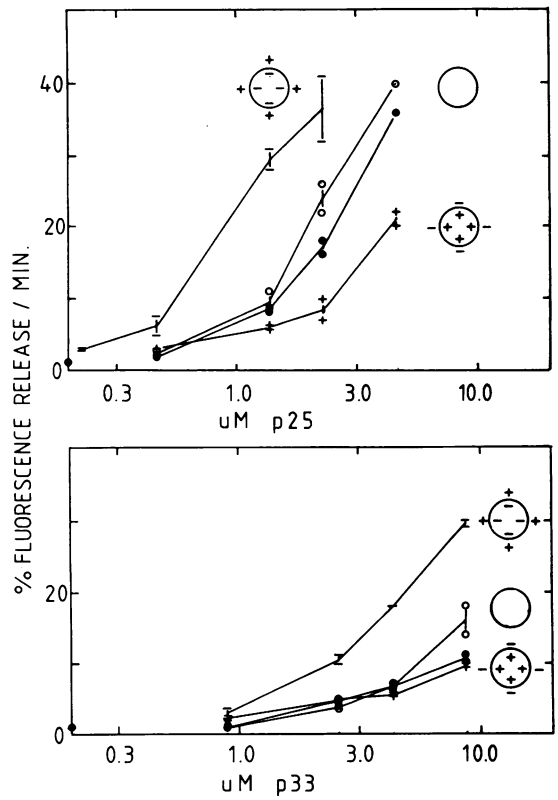

Fig. 4. Rates of vescle disruption by p25 or p33. POPC:CL vesicles loaded with carboxyfluorescein and either Na^+ or K^+ as counter-ions were treated with valinomycin and diluted into a buffered peptide solution containing either Na^+ or K^+ ions. Vesicle disruption was measured by fluorescence dequenching as in Figure 3. Dequenching corresponding to 100% disruption of vesicles was determined upon addition of Triton X-100 to 0.1%. Rates were measured from 30 to 60 s after addition of vesicles to the peptides. (−) K^+-containing vesicles in Na^+ buffer (potential negative inside); (○) K^+-containing vesicles in K^+ buffer (no diffusion potential); (●) Na^+-containing vesicles in Na^+ buffer (no diffusion potential); (+) Na^+-containing vesicles in K^+ buffer (potential positive inside). The presence and the polarity of the K^+-diffusion potential under these conditions is illustrated with each plot.

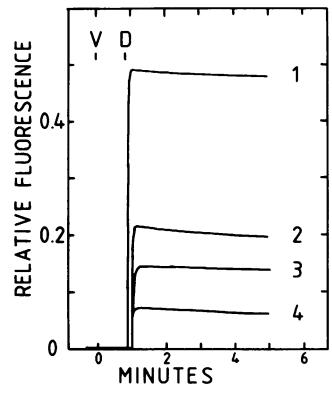

Fig. 5. Demonstration of vesicle membrane potential. POPC:CL vesicles loaded with K^+ carboxyfluorescein (310 mM K^+) and treated with valinomycin were added to solutions containing various K^+ concentrations in a stirred fluorescence cuvette at the point labeled 'V'. No peptides were present. At the point labeled 'D', the potential-sensitive dye, diS-C_3-(5), was added to 1.0 μM, and the fluorescence of the dye (excitation: 620 nm, emission: 670 nm) was followed versus time. **Curve 1.** Dye fluorescence alone; no vesicles added. **Curve 2.** Vesicles plus dye in 310 mM K^+. Quenching compared with **curve 1** is due to dye binding to lipid (Sims *et al.*, 1974). **Curve 3.** Vesicles plus dye in 31 mM K^+. **Curve 4.** Vesicles plus dye in 3.1 mM K^+. In each case, Na^+ was used to equalize the final cation concentration to 310 mM.

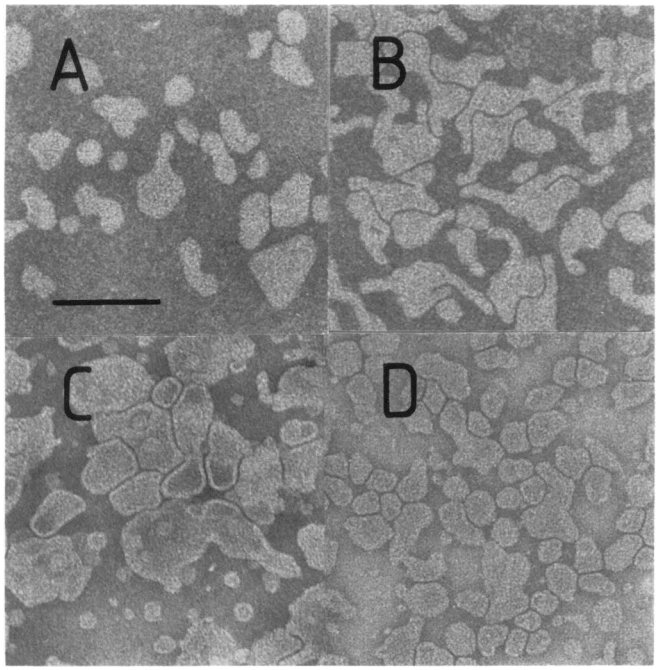

Fig. 6. Electron micrographs of negatively stained POPC:CL vesicles treated with peptide solutions. **(A)** Control vesicles (no peptide). **(B)** p15-treated vesicles. **(C)** p25-treated vesicles. **(D)** p33-treated vesicles. Bar in **A** represents 1000 Å.

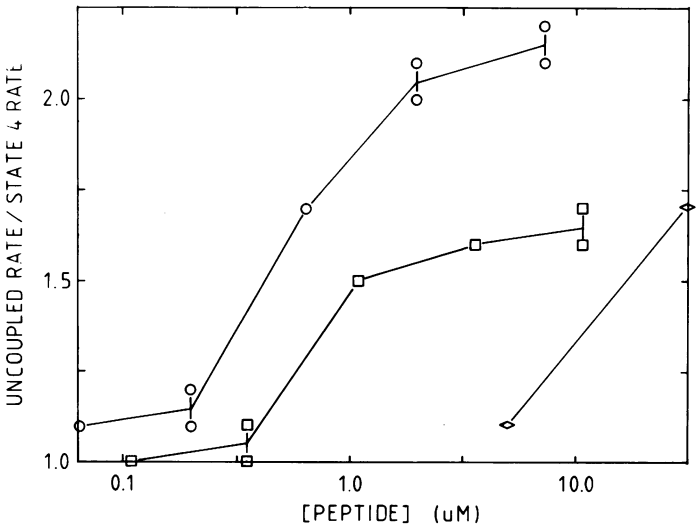

Fig. 7. The peptides uncouple respiratory control in yeast mitochondria. Freshly isolated yeast mitochondria respiring in state 4 with succinate as substrate were treated with various concentrations of the peptides. The maximum respiratory rate observed after addition of the peptide (uncoupled rate) was divided by the rate measured before peptide addition (state 4 rate) and plotted versus the peptide concentration. (○) p33; (□) p25; (◇) p15.

in dye fluorescence with valinomycin-treated, K^+-loaded vesicles which had been diluted into buffer of decreasing K^+ concentration. Increasing potentials, negative inside, led to an increased association of the cationic dye with vesicles which revealed itself as increased quenching of the dye's fluorescence.

Morphology of vesicles treated with pre-sequence peptides

Vesicles were incubated with the peptides in the absence of valinomycin or K^+ concentration gradients. Electron micrographs of negatively-stained vesicles showed morphological

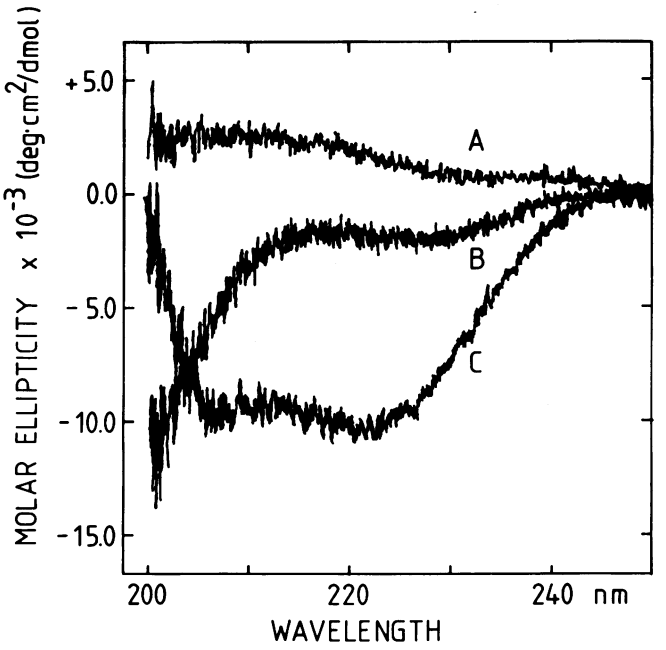

Fig. 8. C.d. of p25. C.d. spectra of p25 solutions in 0.3 M NaCl **(B)** and in 10 mM SDS, 0.3 M NaCl **(C)**. Peptide concentration was 0.04 mg/ml. **Curve A** is 10 mM SDS, 0.3 M NaCl in the absence of peptide.

changes induced by the peptides (Figure 6). Treatment with p15 resulted in aggregation and a slight change in shape of the vesicles compared with the control (panel B versus panel A). Treatment of vesicles with p25 caused massive aggregation and increase in size of the vesicles (panel C). The p33 peptide also caused aggregation of the vesicles (panel D) and at higher peptide-to-lipid ratios caused breakdown of the vesicle structure (not shown).

Pre-sequence peptides abolish respiratory control of isolated yeast mitochondria

'Uncoupling ratios' for various concentrations of each peptide are given in Figure 7. The two longer peptides showed similar concentration dependence for uncoupling of respiratory control, but the p33 peptide actually stimulated the rate of respiration in the uncoupled state. The 15-residue peptide had a significantly weaker effect.

Secondary structure of p25

Circular dichroism (c.d.) spectra were measured to assess the secondary structure of p25. In 0.3 M NaCl, p25 had little secondary structure (Figure 8, curve B). However, in the presence of SDS above the detergent's critical micelle concentration (Helenius and Simons, 1975), marked α-helicity was induced (curve C). Detergent micelles were used as a surface model, since solutions of sonicated unilamellar vesicles became turbid in the presence of p25 and c.d. spectra could not be recorded. An α-helix content of 40–50% was calculated for p25 in SDS from the molar ellipticity change at 222 nm (Chen *et al.*, 1974). This could mean either that only 40–50% of the peptide molecules associate with the micelles but each is 100% helical, that all of the peptides associate with the micelles but each is only 40–50% helical or that there is some intermediate situation.

Discussion

The molecular mechanism of protein import into mitochondria is not known, but some features important in the process have been determined. The best understood component of the system

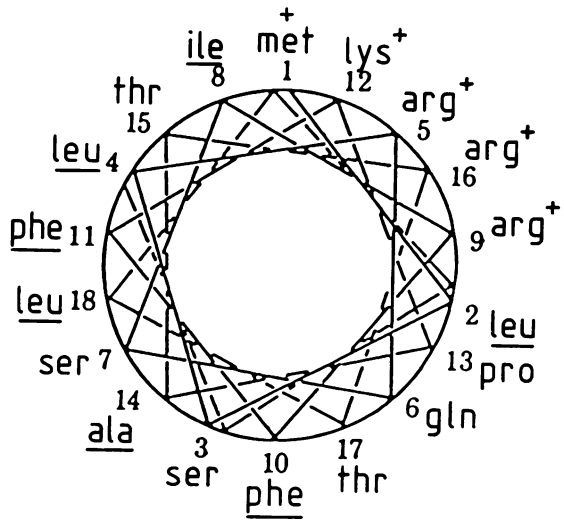

Fig. 9. The pre-sequence of yeast cytochrome oxidase subunit IV can form an amphiphilic helix. Amino-terminal residues 1 – 18 were plotted on a 'helical wheel' as described by Schiffer and Edmundson (1967). Hydrophobic residues are underlined.

is the imported precursor protein itself. Gene fusion experiments have shown that all information required to direct a cytoplasmically synthesized precursor protein to and into mitochondria can be contained in a small amino-terminal region of the protein (reviewed by Hurt and van Loon, 1986). Since a mitochondrial pre-sequence can transport an attached cytosolic protein into mitochondria, it can act independently of its normally attached mature mitochondrial protein. Thus, it probably constitutes a separate structural domain in the precursor polypeptide.

Pre-sequences function in at least two steps during mitochondrial protein import. The first step is binding; precursors whose pre-sequences have been removed are unable to associate with the outer membrane of mictochondria and are not imported (Riezman *et al.*, 1983; Hurt *et al.*, 1985; van Loon and Young, 1986). The second step is energy-dependent translocation. Schleyer and Neupert (1985) have shown that the pre-sequence seems to be the region of a precursor protein which requires an energized membrane for its trans-bilayer movement. The attached 'mature' part of the protein can subsequently be chased into the mitochondrion in the absence of an energized membrane. The results presented here suggest a mechanism by which pre-sequences may effect these functions.

Although pre-sequences have no obvious primary sequence homology, they do display common characteristics as a group. They are generally highly basic, contain no acidic residues and have a high percentage of leucine, serine and arginine (von Heijne, 1986). The regular spacing of the positive charges along the primary sequences suggests a common secondary structure. If the pre-sequence of cytochrome oxidase subunit IV is plotted on a 'helical wheel' (Schiffer and Edmundson, 1967), all positive charges are clustered on one side, whereas uncharged and hydrophobic residues are clustered on the opposite side (Figure 9). Such an 'amphiphilic helix' has been invoked as the key structural feature of many surface-acting peptides (Kaiser and Kézdy, 1983, 1984; Eisenberg, 1984). As discussed in the accompanying paper by von Heijne (1986), most of the mitochondrial pre-sequences that have been sequenced so far fit this model fairly well. C.d. measurements on the synthetic subunit IV pre-sequence (Figure 8) indicate than an α-helical conformation is in fact induced when the peptide is presented with an appropriate surface, such as a detergent micelle. The common property of mitochondrial pre-sequences may thus be their ability to fold into a surface-active amphiphilic α-helix. A similar structural and functional comparison has been made for leader sequences in a bacterial system (Briggs and Gierasch, 1984; Briggs *et al.*, 1985).

The unusual properties of amphiphilic helices result only from the uneven distribution of hydrophobic and hydrophilic residues around the helix, and are relatively independent of the primary sequence of the peptide (for review, see Kaiser and Kézdy, 1983, 1984). One characteristic feature is the structure's affinity for hydrophobic – hydrophilic interfaces. The surface activity of the subunit IV pre-sequence peptides was 10 – 12 mN/m at an air – water interface, and showed even stronger interactions with a monolayer of total extracted mitochondrial phospholipids (Figure 2). The extrapolated critical pressures of insertion for the peptides (43 – 49 mN/m) were even higher than those determined for bee venom melittin or *Staphylococcus aureus* δ-lysin, two similarly sized, highly charged peptide toxins also thought to interact with lipids as amphiphilic helices (Bougis *et al.*, 1981; Bhakoo *et al.*, 1982). The large surface pressure increases can only be explained as insertion of hydrophobic regions of the peptides into the monolayer. First, a simple charge – charge interaction between the highly basic peptides and the phospholipid head groups is unlikely since polylysine of similar mol. wt had no such effect (not shown). Second, although the peptides alone displayed some surface activity, the effect was increased greatly in the presence of lipid: the peptides do not simply push the lipids out of the way (i.e. to reach an air – water interface), they actively associate with them.

A lipid monolayer is limited as a model system since it only simulates half of a biological membrane. For this reason, the experiments with phospholipid vesicles were performed. The results of these experiments (Figures 3 and 4) are significant for two reasons. First, they show that the peptides not only bind to lipid membranes, but can also disrupt them. The ability of a pre-sequence to cause a local defect in mitochondrial membranes could help create a route for the attached mature protein to follow. Second, the enhancement of the lytic effect by a membrane potential, negative inside, immediately recalls the fact that a transmembrane potential of the same polarity across the inner membrane is required for translocation of precursors across that membrane (Schleyer *et al.*, 1982; Gasser *et al.*, 1982; Pfanner and Neupert, 1985). It is not known whether a pre-sequence can sense a potential from outside a mitochondrion, but it has been propsed that close contacts between the inner and outer mitochondrial membranes could lead to a significant potential drop even across the outer membrane (Manella, 1985).

The morphology of vesicles treated with the peptides (Figure 6) gives further evidence of the disruptive effects of the peptides on phospholipid bilayers. Although the micrographs show only a static view of the peptides' actions, and despite the possibility that the membrane structure was altered by the staining process, it seems likely that at least p25 causes membrane fusion prior to vesicle lysis. Such a sequence of events has been established for the interaction of calcium with negatively-charged liposomes: a rapid, initially leak-free fusion of the liposomes is followed by slower release of the liposome contents (Wilschut and Hoekstra, 1984). Melittin and the amino-terminal peptides of certain viral coat proteins also cause fusion of membranes (Eytan and Almary, 1983; White *et al.*, 1983). With the viral proteins, fusogenic activity is a key element in the transfer of viral contents into the invaded cell's cytoplasm (White *et al.*, 1983). For mitochondrial import, temporary fusion of inner and outer mem-

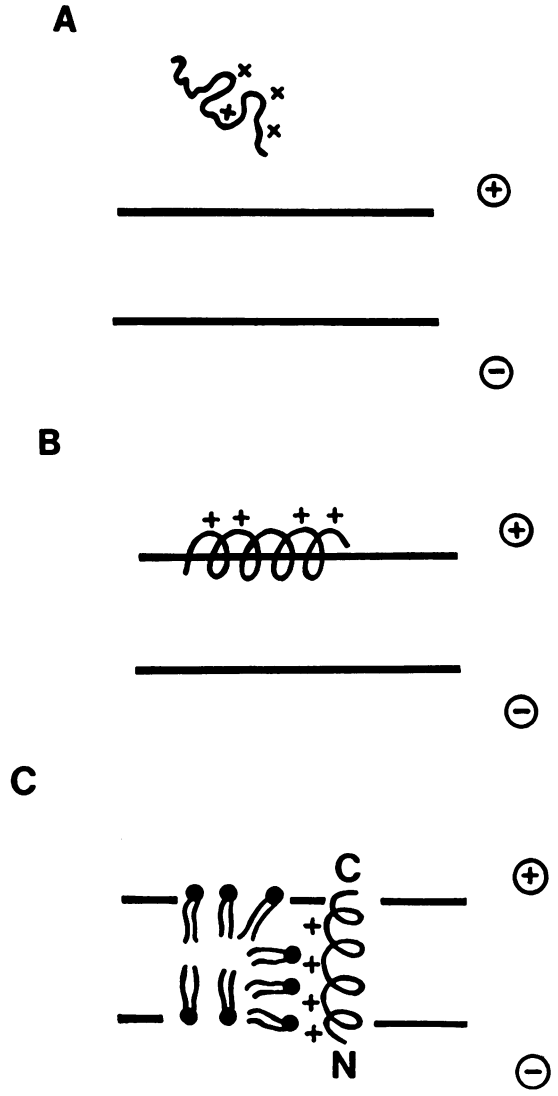

Fig. 10. Possible steps in the interaction of a mitochondrial pre-sequence with an energized mitochondrial membrane (see text).

branes could create an entry point for proteins to the matrix space.

The import of apocytochrome *c* has been well studied and seems to occur via a unique mechanism. This intermembrane space localized protein lacks a cleavable pre-sequence and can be imported into isolated mitochondria in the absence of an energized inner membrane (Harmey and Neupert, 1985). Previous authors have pointed out that apocytochrome *c* can spontaneously insert into, and through, phospholipid bilayers (Rietveld and de Kruijff, 1984; Dumont and Richards, 1984). It will be interesting to learn whether the membrane-interacting domains of that protein correspond to the (as yet unknown) domains which target it into mitochondria, and whether those regions also behave like the peptides described here.

While the present findings with model systems are intriguing, caution should be exercised in extrapolating them to mitochondrial protein import *in vivo*. First, the peptides could have different properties when covalently attached to a transported protein. In fact, our results with the 15-residue peptide showed that it had much weaker lytic properties than the two longer peptides, despite the finding that a fusion protein consisting of only the amino-terminal 12 residues of the cytochrome oxidase subunit IV pre-sequence attached to mouse dihydrofolate reductase could be imported into mitochondria (Hurt *et al.*, 1985). This could indicate that the function of a pre-sequence is modulated by its attachment to the mature protein. Second, the liposomes tested in this study contain phospholipid alone. Evidence has been presented for the involvement of a proteinaceous receptor in protein import (Gasser *et al.*, 1982; Riezman *et al.*, 1983; Zwizinski *et al.*, 1984), and the actual mechanism of import may be a combination of interactions between the pre-sequence and lipids and/or between the pre-sequence and receptor proteins on or in the mitochondrial membranes. The results presented here open the possibility that the function of a proteinaceous 'receptor' in mitochondrial protein import may, however, not mediate the initial interaction between the precursor protein and a mitochondrion. Since the pre-sequences can sense a potential across a membrane bilayer, *trans* negative (this work), and since the mitochondria present the only such surface to the cytoplasm in non-plant cells [mitochondria are stained specifically *in vivo* by cationic lipophilic rhodamine dyes (Johnson *et al.*, 1980)], specific targeting of precurosor proteins to the mitochondria may occur entirely via the pre-sequences. Alternative requirements for proteinaceous components of an import machinery may occur at the level of enzymatic modification of imported proteins during the import process, or in the formation of an internal import 'pore' to bridge the mitochondrial membranes.

A simple working model emerging from this and other studies is outlined in Figure 10. The pre-sequence, free in the cytoplasm and lacking significant secondary structure, is attracted to the mitochondrial surface through electrostatic interaction either with the membrnae potential or with negatively charged phospholipid head groups (A). An amphiphilic helix then forms whose hydrophobic surface buries into, and possibly destabilizes, the outer leaflet of the outer membrane (B). The helix is then somehow inserted into the membrane by the membrane potential, either through electrophoresis of the positive charges, orientation of the helical dipole in the direction of the electrical field or a combination of the two effects (C). As more fully discussed in the accompanying paper by von Heijne (1986), the energy gain of this step (or steps) could at least partly offset the energy requirement for locally disorienting and moving charged groups through the bilayer. The pathway of the import after this step is unclear, but fusion of the inner and outer membrane at some point in the process could complete the transfer of the pre-sequence into the mitochondrial matrix. While this model ignores the fate of the mature portion of the imported protein, this seems permissible since transfer of the pre-sequence into the matrix can precede import of the rest of the protein (Schleyer and Neupert, 1985). Further support for the model comes from Kellems *et al.* (1975), who showed by electron microscopy that import of mitochondrial proteins may occur through sites of close contact between the inner and outer membrane. If pre-sequences were attracted to such sites, or could induce their formation (possibly by membrane fusion), transfer of a protein through the mitochondrial double membrane system would be greatly simplified.

The surface-active properties of the subunit IV pre-peptide, the effect of a membrane potential on this activity and the potential amphiphilic properties of other mitochondrial pre-sequences could all be adventitious, but we find this difficult to accept. Indeed, these features appear uniquely suited to explain how mitochondrial pre-sequences guide attached proteins into mitochondria. The model experiments reported here allow specific predictions which can be tested under conditions which more closely approach those of living cells.

Materials and methods

Materials

Synthetic palmitoyl oleoyl phosphatidyl choline (POPC) was from Avanti Polar Lipids, Inc. (USA), and bovine brain cardiolipin (CL) was from Sigma. Crude 6-carboxyfluorescein was obtained from Eastman-Kodak and was purified by the method of Ralston *et al.* (1981) prior to use.

Peptide synthesis

The peptides were synthesized by an improved solid-phase method which minimizes many of the side reactions associated with conventional solid-phase methods (Hunkapillar *et al.*, 1984; Kent, 1980). Yields in excess of 99.8% per coupling cycle were obtained when the purest commercial BOC-amino acids, solvents and the chemically stable PAM-resin (Kent, 1984) were used in conjunction with the following accelerated manual synthesis protocol. PAM-resin substituted with 1.0 mmol/g resin of the desired C-terminal BOC-amino acid was purchased from Applied Biosystems, Inc. The side-chain protected BOC-amino acids (Peninsula Laboratories) were reacted as either pre-formed symmetric anhydrides or hydroxybenzotriazole esters (for glutamine and asparagine). Incorporation of each amino acid was performed as follows: (i) the resin was washed six times with dichloromethane (DCM); (ii) the amino-protecting BOC-group was removed with 65% trifluoroacetic acid (TFA) in DCM (10 min reaction); (iii) the resin was flow-washed for 2 min with DCM; (iv) the amino group was neutralized with diisopropylethylamine (twice for 1 min each); (v) the resin was again flow-washed for 2 min with DCM; (vi) BOC-amino acid:dicyclohexylcarbodiimide (2:1 relative to resin-bound amino acid) in DCM was added for 10 min, followed by addition of dimethylformamide (to 50%) for an additional 60 min of reaction; (vii) progress of the coupling was monitored by the quantitative ninhydrin test (Sarin *et al.*, 1981); (viii) the coupling was repeated (from step iii) if the reaction was incomplete; (ix) resin was again flow-washed for 2 min with DCM; (x) the cycle was repeated for the next amino acid.

The completed peptides were cleaved from the resin and deprotected in anhydrous HF (90%) in the presence of *p*-cresol and *p*-thiocresol for 30 min at 0°C. The peptides were precipitated with diethyl ether and separated from the resin by dissolving the peptide precipitate in 5% acetic acid followed by filtering and lyophilization. The peptides were analyzed by Edman degradation using an Applied Biosystems gas-phase protein sequenator, as well as by reverse-phase h.p.l.c. on a Vydac C-4 column using 0.1% TFA in a water/acetonitrile gradient. The overall yield of the peptides was >92% as shown by integration of the h.p.l.c. peak areas. The peptides were further gel-filtered on Biogel P-2 in 100 mM ammonium acetate and lyophilized prior to use.

Peptide concentrations

Concentrations of peptide solutions were determined by quantitative amino acid analysis. For each peptide solution, absolute concentration values were then converted to absorbance values obtained by the method of Lowry *et al.* (1951). This method was then used for routine experiments.

Isolation of mitochondrial phospholipids

Yeast mitochondria were isolated from strain D273-10B grown on 2% lactate as the major carbon source as described by Daum *et al.* (1982). Total lipids were extracted from the mitochondria by the method of Bligh and Dyer (1959). Phospholipids were subsequently prepared by precipitation from ice-cold acetone. The phospholipids thus isolated were re-dissolved in chloroform and stored under nitrogen at −20°C. T.l.c. on silica gel ($CHCl_3$:CH_3OH:H_2O, 65:25:4) revealed the expected mixture and relative amounts of the various phospholipids (Daum, 1985).

Lipid monolayers

Lipid monlayer experiments were performed on a multicompartment Langmuir trough (Model RMC2-T, Mayer Feintechnik, Göttingen, FRG) using a single section of the trough. The monolayer surface area was held constant at 40 cm^2 throughout. Water used had been ion-exchanged and glass distilled. Surface pressures were measured by the Wilhelmy method using plates cut from Whatman No. 1 filter paper and rinsed with methanol prior to use (Fromherz, 1975). 25 ml of 5 mM KP_i, pH 7.0 was added to the trough, and the surface was cleaned by careful aspiration as the Teflon barriers were brought together. After readjusting the surface area and adjusting the surface pressure to zero, the mitochondrial lipids (~10 μg in 10 μl hexane:ethanol, 9:1) were slowly applied to the surface with a syringe. When the desired initial pressure was reached, the solvent was allowed to evaporate for 5 min. Peptide solutions (up to 25 μl in water) were then injected through the monolayer into the aqueous subphase, and the surface pressure increase of the monolayer was measured as a function of time. The subphase was stirred by a small magnetic stirring bar. Final pressure values were normally reached within 15 min after peptide addition. All experiments were performed at room temperature.

Vesicle preparation

All vesicles were prepared from a mixture of synthetic POPC and bovine brain CL (2:1, based on phospholipid phosphorus). The lipids (in chloform) were dried on the walls of a 25 ml round-bottom flask by rotary evaporation, and further dried for at least 1 h under high vacuum. For the carboxyfluorescein-loaded vesicles, a solution of 50 mM carboxyfluorescein, 75 mM tartrate, 10 mM Hepes pH 7.2 (all as either Na^+ or K^+ salts) was added to the lipids so as to give a final concentration of 20 mM phospholipid phosphorus. The solution was sparged with nitrogen, and when the lipid was maximally dispersed, the sample was sonicated in an Elgasonic bath sonicator (Elga AG, Biel, Switzerland) for 15 min at room temperature. The solution normally cleared within 5 min. Unencapsulated carboxyfluorescein was removed by gel filtration on Sephadex G-50 (coarse grade) equilibrated with 150 mM tartrate, 10 mM Hepes pH 7.2 (Na^+ or K^+ salts depending on the buffer entrapped within the liposomes). For electron microscopy, vesicles were prepared in exactly the same way, except that (i) 150 mM K^+ tartrate, 10 mM K^+ Hepes pH 7.2 was used to hydrate the lipid, and (ii) the smallest vesicle population was isolated by size on a Sepharose 4B column (40 × 1.0 cm) equilibrated with 150 mM K^+ tartrate, 10 mM K^+ Hepes pH 7.2 (Huang, 1969). Phospholipid concentrations were determined as total phosphate after ashing (Ames and Dubin, 1960).

Vesicle lysis

A suspension of carboxyfluorescein-loaded vesicles (containing 6.0 mM phospholipid phosphorus) was treated with 10 μM valinomycin. Lysis was measured directly in 2.5 ml fluorescence cuvettes containing peptide solutions in either 150 mM Na^+ tartrate, 10 mM Na^+ Hepes pH 7.2 or 150 mM K^+ tartrate, 10 mM K^+ Hepes pH 7.2. Vesicles were diluted 1:100 into the assays (final phospholipid concentration: 60 μM, final valinomycin concentration: 0.1 μM) and fluorescence increse was measured on a Schoeffel RRS 1000 recording fluorometer thermostatted at 20°C and equipped with a stirring motor. Excitation was at 490 nm and emission at 525 nm.

Measurement of membrane potential

The relative magnitude of the diffusion potential induced by valinomycin in K^+-loaded vesicles at various external K^+ concentrations was measured as described in the legend to Figure 5 using the method of Sims *et al.* (1974).

Electron microscopy

Peptide solutions (10 μM final concentration) were added to suspensions of POPC:CL vesicles (0.4 mM final phospholipid phosphorus concentration) in 150 mM K^+ tartrate, 10 mM K^+ Hepes pH 7.2. After 20 min at room temperature, the samples were treated for 1 min with 2% K^+ phosphotungstate on carbon-coated grids that had been treated by glow-discharge. Microscopy was performed with a Philips 300 microscope operated at 80 kV.

Mitochondrial uncoupling

Yeast mitochondria were isolated from lactate-grown cells as described above. Respiratory control was measured polarographically using the conditions of Yaffe *et al.* (1985) with 10 mM Na^+ succinate as substrate. After a short period of state 3 respiration (induced by an initial pulse of 0.1 μmol ADP) had subsided, peptide was added. The respiratory control ratio (state 3/state 4) of the mitochondria was routinely between 1.8 and 2.0, and the P/O ratio was close to 2.0, as expected for succinate.

Circular dichroism

C.d. was measured with a modified version of a Cary 61 Circular Dicrograph (Baechinger *et al.*, 1979). The instrument was calibrated with *d*-(+)-camphorsulphonic acid, and all measurements were performed at 20°C.

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